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Pectin plays an important role on the kinetics properties of polyphenol oxidase from honeydew peach

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ABSTRACT

Polyphenol oxidase (PPO) was purified from peach pulp by a three-step column chromatographic procedure. The kinetics properties of the PPO fractions obtained from different purification steps were compared. All the fractions showed high affinities for (+)-catechin and (−)-epicatechin. The optimum pHs and optimum temperatures for all the fractions were the same. However, the fraction that contained pectin was more sensitive to the change of pH, and it had a lower affinity for the substrates and a higher thermostability than the fractions without pectin. In addition, the protein impurities in PPO fractions might have no effect on the properties of PPO. L-Cysteine and glutathione were effective for the inhibition of all the PPO fractions, while NaF inhibited moderately. However, the pectin could reduce the inhibition effects of those inhibitors.

1. Introduction

Honeydew peach (*Prunus persica* L. *Batsch*) has an increasing market value due to its sensory and nutritional qualities, as well as multiple biological effects. However, browning is an important problem that limits the development of honeydew peach juice and the canning industry. Polyphenol oxidase (PPO) is thought to be one of the major factors contributing to the browning of fruits and vegetables. Enzymatic browning is caused by the oxidation of phenolic substrates by PPO to produce reactive quinones. These quinones are highly reactive species involved in various reaction pathways. They are powerful electrophiles which may suffer nucleophilic attack by other polyphenols, amino acids or proteins to produce dark-brown or black pigment in senescent and postharvested fruits and vegetables (Cabanes, García-Cánovas, & García-Cármona, 1987; Fulcrand, Cheminat, Brouillard, & Cheynier, 1994; Hurrel & Finot, 1984).

In order to reveal the mechanisms and find methods for the control of the enzymatic browning of fruits and vegetables, numerous studies had been focused on the characterisation of PPO from different plant resources. Most researchers have used a crude extract or a partial purified enzyme to characterise the PPO from different sources (Chutintrasri & Noomhorm, 2006; Dogan & Dogan, 2004; Jiang, Zauberman, & Fuchs, 1997; Jiménez-Atiénzar, Cabanes, Gandía-Herrero, & García-Carmona, 2004; Liu et al., 2007; Nagai & Suzuki, 2001; Núñez-Delicado, Serrano-Megías, Pérez-López, & López-Nicolás, 2007). In recent years, more and more reports focused on the characterisation of PPO using a purified PPO (Arslan, Erzengin, Sinan, & Ozensoy, 2004; Gao, Han, & Xiao, 2009; Mishra, Gautam, & Sharma, 2012; Sellés-Marchart, Casado-Vela, & Bru-Martínez, 2006; Sun et al., 2012). However, results obtained using crude PPO, partially purified PPO and purified PPO, whose properties are closest to the properties of PPO in the juice or fruit tissue, were ambiguous.

In our previous studies, it was found that the PPO in the peach juice was more stable than the PPO extract in the buffer solution system. This meant that some matters, such as pectin and cellulose

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contained in the peach juice, could influence the properties of PPO. Moreover, the pectin-free PPO extract was difficult to obtain from peach, due to the high pectin content, and it was precipitated together with PPO during the ammonium sulphate fractional precipitation process. To remove the pectin from PPO, column chromatographic purification should be further employed. However, as far as we know, there is no information available in the literature about the effect of pectin on the properties of PPO, and the differences between the properties of purified PPO and crude PPO are ambiguous. Thus, the purpose of the present work was to compare the kinetics properties among the PPO obtained from different purification steps. Results from this study will be helpful to develop methods for the control of the enzymatic browning in the processing of peach.

2. Materials and methods

2.1. Materials

The peaches (*Prunus persica* L. *Batsch* cv. Yulu) at commercial maturation were obtained from Ningbo, Zhejing Province. The fruits were peeled and stoned, and then stored at −20 °C until extraction. Biochemicals were purchased from Sigma and all other chemicals were of analytical grade.

2.2. Extraction of PPO from peach pulp

Peach pulp (5000 g) was homogenised with acetone (9000 ml) (−20 °C) for 1 min, and the mixture was refrigerated for 2 h, before filtration. The residue was rinsed with acetone (5000 ml) (−20 °C) to eliminate phenolic compounds, and the PPO was in the residue. The residue was laid in the fume hood to remove the residual acetone, and then the residue (acetone powder) was collected. Acetone powder of peach pulp and insoluble polyvinylpyrrolidone (PVPP, 5%) were homogenised in 100 mM phosphate buffer (400 ml) (pH 6.8) for 20 min and then the homogenate was centrifuged at 8000*g* for 5 min. The supernatant was pooled and subjected to the ammonium sulphate fractional precipitation and column chromatographic purification, respectively.

2.3. Ammonium sulphate fractional precipitation (protocol 1)

Solid ammonium sulphate was added to the supernatant, and the precipitate obtained between 40% and 80% saturation was collected by centrifugation at 8000*g* for 10 min, and then the precipitate was dissolved in 50 mM phosphate buffer (pH 6.8). The enzyme was dialyzed against the same buffer at 4 °C overnight.

2.4. Column chromatographic purification (protocol 2)

2.4.1. Hydrophobic chromatography on Phenyl-Sepharose CL-4B

The supernatant was supplemented with solid ammonium sulphate to a final concentration of 40% saturation (1.84 M), and the supernatant was collected by centrifugation at 8000*g* for 10 min, and then chromatographed on a Phenyl-Sepharose CL-4B column (2 cm × 20 cm). The column was washed with 50 mM phosphate buffer (pH 6.8) containing 1.84 M ammonium sulphate and then eluted with 50 mM phosphate buffer (pH 6.8) at a flow rate of 2 ml/min.

2.4.2. Ion-exchange chromatography on DEAE Sepharose fast flow

The enzyme fraction obtained from Phenyl-Sepharose CL-4B column was dialyzed against 10 mM Tris–HCl buffer (pH 6.8) at 4 °C overnight, and then loaded onto a DEAE Sepharose Fast Flow column (1.6 cm × 10 cm). The column was washed with 10 mM Tris–HCl buffer (pH 6.8) and then eluted with 50 mM Tris–HCl buffer (pH 6.8) at a flow rate of 1.5 ml/min.

2.4.3. Size-exclusion chromatography on Sephaoryl HR S-200

The enzyme fraction obtained from DEAE Sepharose Fast Flow column was loaded onto a Sephaoryl HR S-200 column (2.0 cm × 70 cm) equilibrated with 50 mM phosphate buffer buffer (pH 6.8). The column was washed with the same buffer at a flow rate of 0.5 ml/min.

2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to the method of Laemmli (1970). The PPO samples were denatured by boiling with mercaptoethanol and separated in a 10% polyacrylamide gel. The gels were stained with Coomassie brilliant blue R250.

2.6. Enzyme activity and protein content assay

PPO activity was assayed spectrophotometrically at 25 °C by monitoring at 435 nm. The reaction medium (3 ml) contained 1 ml of 3 mM (+)-catechin, 1.9 ml of 50 mM of phosphate buffer (pH 6.8), and 0.1 ml of the enzyme solution. One unit of enzyme was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min at 25 °C.

The protein content was determined according to Bradford's dye binding method, using bovine serum albumin (BSA) as a standard (Bradford, 1976).

2.7. Determination of pectin content

Determination of pectin content was performed according to the method described previously with some modifications (Wang, Chuang, & Hsu, 2008). Briefly, 0.5 ml of sample solution was added to 6 ml of 0.0125 M sodium tetraborate (in concentrated sulfuric acid) and then heated for 5 min in a boiling water bath. Colour development followed addition of 0.1 ml of 0.15% *m*-hydroxydiphenyl and incubation for 20 min at room temperature. NaOH (0.1 ml) was added instead of 0.15% *m*-hydroxydiphenyl to the control. Pectin contents were expressed as galacturonic acid equivalents.

2.8. Enzyme kinetics and substrate specificity

The activity of different PPO fractions were assayed using (+)-catechin, (−)-epicatechin and diphenols catechol as substrates, and the rates of the reactions were measured at various substrate concentrations at the maximum absorption wavelength for the corresponding chromophore ((+)-catechin (435 nm), (−)-epicatechin (430 nm) and diphenols catechol (398 nm)). The Michaelis constant (K_m), maximum velocity (V_{max}) and specificity (V_{max}/K_m) for the different substrates were calculated from a plot of the reciprocal of initial velocity ($1/V$) versus the reciprocal of substrate concentration ($1/[S]$) according to the method of Lineweaver and Burk.

2.9. Effect of pH on PPO activity

Optimum pH for the activity of different PPO fractions were determined in the pH range of 3.0–10.0 by using 50 mM citrate (pH 3.5–5.0) and 50 mM phosphate (pH 6.0–8.0) buffer at 25 °C. The reaction medium (3 ml) contained 1 ml of 3 mM (+)-catechin, 1.9 ml of 50 mM of buffer (different pH values), and 0.1 ml of the enzyme solution. The initial velocity was determined at 390 nm (pH 3.5–6.0) or 435 nm (pH 6.5–7.5).